Laboratory Evaluation of Avian Odors for Mosquito (Diptera: Culicidae) Attraction

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ABSTRACT Attraction of *Culex quinquefasciatus* Say, *Culex tarsalis* Coquillett, *Culex nigripalpus* Theobald, and *Aedes aegypti* (L.) to avian and other host odors was investigated in a dual-port olfactometer. Although attraction to a human arm was high for *Ae. aegypti* (>80%) and low for all *Culex* spp. (<25%), all species responded similarly to a chicken (55.3–73.6%). Responses of *Ae. aegypti*, *Cx. quinquefasciatus*, and *Cx. nigripalpus* to feathers were low (<20%) but greater than to controls. There was no difference in attraction of *Cx. tarsalis* to feathers or controls. Responses to CO_2 (5 ml/min) were low for all species (<15%) except *Cx. tarsalis*, which were moderate (24.5%). When feathers were combined with CO_2, the resulting attraction was additive or lower than responses to feather and CO_2 alone for all species except for *Cx. tarsalis*, which had responses that were three-fold greater than expected if responses were additive. The CO_2–feather treatments were less attractive than a chicken for all species. When olfactometer assays were extended from 3 to 20 min, responses by *Ae. aegypti* significantly increased to a chicken and CO_2 and attraction of *Cx. quinquefasciatus* significantly greater to chickens, CO_2, and feathers. None of the volatile compounds previously identified from feathers or uropygial glands tested were attractive. Both feather-rubbed cotton balls and hexane extracts of feathers were attractive and as attractive as feathers; however, ether extracts were not attractive. Feathers clearly contribute to the attraction of host-seeking *Culex* spp., and future studies will focus on identification of the attractant compounds.

KEY WORDS host attraction, birds, *Culex*, carbon dioxide

ALTHOUGH BIRDS PLAY A CRITICAL role in maintenance and amplification of mosquito populations and as reservoirs of pathogens that affect humans and other animals, little is known about the cues used by mosquitoes to locate birds. Most traps use an attractant such as CO_2 that is effective for anthropophilic species such as *Aedes aegypti* (L.) and a range of other species (Service 1993). Additional attractants such as the bovine breath component 1-octen-3-ol enhance collection of a broader range of species (Kline et al. 1991, Kline 1994, Kline and Mann 1998). Animal-baited traps provide specific volatile cues from hosts for attraction of mosquitoes (Service 1993) and often overcome inherent biases from conventional traps and attractants. For example, baiting traps with birds can result in collections of predominately *Culex* or *Culiseta* spp. (Dow et al. 1964, Ehrenberg 1966, Emord and Morris 1982, Rutledge et al. 2003, Lepore et al. 2004) that are not readily collected in conventional traps (Sudia et al. 1967, Nayar et al. 2001). Because of the sensitivity of these traps, they are valuable in arbovirus surveys and population monitoring (Reeves et al. 1961, Downing and Crans 1977, Rutledge et al. 2003). However, little is known about the volatiles emitted by avians other than the emanation of CO_2 from breath and 2,3-*n*-alkanediols from uropygiol glands (Haahti and Fales 1967). In Gambia, mosquitoes were attracted from a greater distance to avian hosts than to CO_2 alone (Gillies and Wilkes 1974), indicating that volatiles other than CO_2 play an important role in attraction. Recently, Williams et al. (2003) collected and identified several volatile compounds by solid phase microextraction (SPME) from chicken feathers, but these compounds remain untested for behavioral response. The lack of mosquito attractants based upon avian odors is a barrier for development of an avian odor-based lure for traps for use in mosquito population and arbovirus surveillance.

The objective of this study was to compare attraction responses of *Culex* to chickens, compounds identified by Williams et al. (2003) from feathers and diol compounds identified from uropygial glands and extracts of feathers. *Ae. aegypti* (L.) was included in these studies as a representative anthropophilic species.

Materials and Methods

Ae. aegypti, *Culex quinquefasciatus* Say, *Culex nigripalpus* Theobald, and *Culex tarsalis* Coquillett were

reared in the laboratory using methods described by Gerberg et al. (1994). Source and year of establishment for colonies are *Ae. aegypti* (Orlando, FL, 1952), *Cx. quinquefasciatus* (Gainesville, FL, 1995, supplemented by new stock every 1 to 2 yr), *Cx. nigripalpus* (Vero Beach, 1999), and *Cx. tarsalis* (Coachella Valley, CA, 2001). Adults were maintained in screen cages with a 10% sugar solution provided continuously. Cages were held at 27–29°C and 70–85% RH under a photoperiod of 14:10 (L:D) h. For bioassays, unfed females 7–14 d old were used.

Olfactometer. To determine whether treatments elicited an upwind orientation response, unfed female mosquitoes were tested in a triple-cage dual-port olfactometer (Posey et al. 1998). The clear acrylic olfactometer consisted of a large chamber that led to two circular ports upwind of the chamber. Three chambers with test ports were stacked but only one chamber at a time was used for assays. Air flowing through the olfactometer was obtained externally, then charcoal-filtered, humidified, and warmed (27 ± 1°C and 60 ± 2% RH). At the beginning of each test, a door was opened to allow air to flow through the ports (28 ± 1 cm/s) into the chamber. This door, when closed, trapped mosquitoes in the ports so that they could be counted at the end of a test. During a test, mosquitoes in the test chamber could follow an upwind air current to the treatment test port, to the control test port, or remain in the chamber. Each cage was loaded with 50–70 females (7–14 d old) that were collected from stock cages into release chambers by using a draw box (Posey and Schreck 1981) that selectively collected active and responsive females. Once loaded in the chambers, mosquitoes were allowed to acclimate for ≈1 h before testing. Responses were calculated as the percentage of total mosquitoes tested that were trapped in the treatment port or the control port. Treatments and controls were randomly assigned to the left or right ports. All materials placed in the treatment or control ports for testing were handled with gloves to avoid contamination with skin compounds. Each day, mosquitoes from each stock cage used were tested for responsiveness using a hand or CO_2 (5 ml/min) in preliminary olfactometer assays. If responses were below a preset criterion, assays were not conducted. A test consisted of placing treatment and control materials in the respective ports, opening the door to allow air flow over the materials into the test chamber and closing the door at the end of the test. Assays consisted of 12–16 replicates. Tests with *Ae. aegypti* are generally conducted for 3 min or less (Geier and Boeckh 1999, Bernier et al. 2003). However, because preliminary studies indicate that *Culex* were slower in responding, tests were extended to 20 min with observations of the numbers of mosquitoes in the treatment and control port made at 3, 5, 10, and 20 min. Assays with *Ae. aegypti* were conducted under high light conditions (2,220–2,400 lux) between 1000 and 1500 hours. Assays with *Culex* were conducted under low light conditions (100–150 lux) between 1400 and 1900 hours.

Test Materials. Tests were conducted by determining responses of each species to a range of treatments, including a Leghorn hen, human arm, 10 g of freshly collected feathers, CO_2 (5 ml/min), and CO_2 in conjunction with feathers. The rate of carbon dioxide tested was chosen so that additive effects with feathers could be detected more clearly. An unrestrained chicken, *Gallus gallus domesticus* L., was placed in an acrylic box (30.5 by 17.8 by 15.2 cm) with an average flow of 5 cm/s of air filtered, humidified, and warmed as described above. The chicken was allowed to settle (≈5 min) before the test was initiated. Attraction to a human hand was evaluated by placing a hand through the iris diaphragm into an olfactometer port. Hands were not washed within an hour of the test, and contact with chemicals was avoided. The hand did not contact the interior sides of the olfactometer to avoid contamination with skin compounds. Feathers were clipped from the back and sides of chickens and handled using gloves to avoid contamination with human skin oils. Feathers were tested within 1 h of collection. For testing, 10 g of feathers were contained in cotton stockinette, placed on a disposable petri dish, and placed in the treatment port. Each chicken represented an estimated 40–50 g of feathers, and efforts to consistently assay larger volumes of feathers were difficult because of restricted air flow in the test port. Efforts were not made to standardize treatments by weight or surface area but to obtain comparative responses between treatments. Assays consisted of 12 replicates. Use of animals in this research was reviewed and approved (projects D207 and D469) by the University of Florida Institutional Animal Care and Use Committee, Gainesville, FL.

Extracts. Cotton balls (1.6 g) were rubbed on feathers on the back and sides of chickens for 3 min. Three cotton balls were placed in a disposable petri dish (10 cm in diameter) and immediately tested in the olfactometer. Untreated cottons balls were used as controls. Solvent extracts (diethyl ether and hexane) of 10 g of feathers also were made for evaluation. For these extracts, 10 g of feathers were packed into a glass funnel with a glass wool filter, 20 ml of solvent dripped over the feathers and collected in vials below. All extracts were stored at −20°C and before testing volume reduced to 200 μl under N_2. Extracts were placed on watch glasses (5 cm in diameter), and once the solvent was dried, glasses were placed in the test port of the olfactometer. To determine whether CO_2 enhanced the attractiveness of feathers, CO_2 (from a compressed gas cylinder) was added to the airstream containing the feathers. All glassware and glass wool were solvent rinsed (with ether then hexane) before use, and all materials were handled with gloves to avoid contamination with skin oils.

Chemicals. Volatile compounds from feathers evaluated included hexanal, nonanal (Acros Organics, NJ), α-pinene, benzaldehyde, and β-myrcene (Sigma-Aldrich Chemicals, St. Louis, MO). Uropygiols based on Haahti and Fales (1967) were evaluated and included meso-2,3-butanediol, 2,3-butanediol, and 2,3-docosanediol (Bedoukian Research, Danbury, CT).

Compounds (100 μl) were placed in the olfactometer in vial caps (9 mm i.d. by 9 mm in height) with the test compound placed in the treatment port of the olfactometer and an empty cap placed in the control port of the olfactometer. The proportions of these compounds from the SPME analysis of feathers by Williams et al. (2003) was determined an a mixture consisting of 21% hexanal, 12% α-pinene, 12% benzaldehyde, 23% β-myrcene, and 32% nonanal also were tested (100 μl total volume). CO_2 was obtained from a pressurized cylinder, and assays consisted of 12 replicates.

Statistics. A paired *t*-test ($P < 0.05$) was used to compare responses between control and treatments in the olfactometer and also between treatment responses at 3 and 20 min. Before analysis data were arcsine transformed. One-way analysis of variance (ANOVA) was used to determine whether there were differences in responses of different species to arm, chicken, feathers, CO_2, and the feather–CO_2 combination. Means were separated by Student–Newman–Keuls test ($P < 0.05$).

Results

In the absence of odor added to the airstream, few mosquitoes of any species (<2%) entered the treatment or control port of the olfactometer. Mosquitoes responded strongly to odors tested with significant differences in response between species (Fig. 1). For all species and treatments, relatively few mosquitoes (<3%) were collected in the control ports, and all treatments were significantly larger than their corresponding controls ($P < 0.05$), except the responses of *Cx. tarsalis* to an arm ($t = 1.46$, df = 22, $P = 0.07$) and to feathers ($t = 1.67$, df = 22, $P = 0.06$), which did not differ from their corresponding controls.

Attraction to the human arm differed significantly between species of mosquito tested ($F = 36.76$; df = 3, 63; $P < 0.0001$) (Fig. 1). *Ae. aegypti* were strongly attracted to the arm with fewer than a third of *Cx. quinquefasciatus* attracted. The arm was relatively unattractive to *Cx. tarsalis* (<1%) and *Cx. nigripalpus* (<5%) with no difference between these species. In contrast, all species were highly attracted to the chicken (55.3–77.6%) with no differences in attraction between the species tested ($F = 2.33$; df = 3, 63; $P = 0.08$).

Responses to feathers were moderate but consistent with generally <20% attraction for all species. Attraction differed significantly between species ($F = 4.86$; df = 3, 63; $P = 0.004$) with significantly more *Cx. quinquefasciatus* responding to feathers than *Ae. aegypti* (10.8%) and *Cx. tarsalis* (0.9%) (Fig. 1). Responses of *Cx. quinquefasciatus* and *Cx. nigripalpus* were similar (Fig. 1). Collection of mosquitoes in the treatment port with CO_2 also differed significantly between species ($F = 5.64$; df = 3, 63; $P = 0.005$) with significantly more attraction by *Cx. tarsalis* than by other species (Fig. 1). Responses of *Ae. aegypti* and *Cx. quinquefasciatus* to CO_2 were lower (10.8–12.2%) and

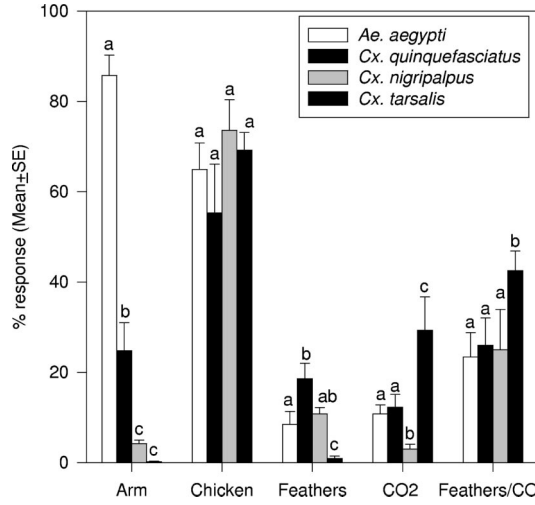

Fig. 1. Responses of host-seeking female mosquitoes to a human arm, a Leghorn hen, chicken feathers, CO_2, and a combination of feathers and CO_2 in 3-min olfactometer assays. Bars represent percentage of total mosquitoes tested that were trapped in the treatment port. Bars with similar letters within each treatment are not significantly different. $n = 12$.

Cx. nigripalpus females exhibited relatively little attraction (<5%).

All mosquito species were moderately attracted to the combination of CO_2 and feathers with significant differences in responses between species ($F = 9.03$; df = 3, 63; $P < 0.001$) (Fig. 1). Responses of *Cx. nigripalpus* and *Cx. tarsalis* (42.5–43.5%) were greater than those of *Ae. aegypti* and *Cx. quinquefasciatus* (23.4–26.0%). Generally, attraction to the CO_2–feather combination was equal to or less than expected if the responses of feathers and CO_2 alone were additive. Attraction responses of *Cx. nigripalpus*, however, were more that three-fold greater than the additive effect of CO_2 alone and feathers alone. In summary, responses to chickens were significantly greater for all species than to feathers, CO_2, or the combination of feathers and CO_2 ($P < 0.05$). Mosquito responses to the chicken were significantly greater than to the CO_2–feather combination for *Ae. aegypti* ($t = 5.49$, df = 30, $P < 0.001$), *Cx. quinquefasciatus* ($t = 5.37$, df = 30, $P < 0.001$), *Cx. nigripalpus* ($t = 5.40$, df = 30, $P < 0.001$), and *Cx. tarsalis* ($t = 3.68$, df = 30, $P < 0.05$).

When the time duration of olfactometer assays was extended, significantly more *Ae. aegypti* were collected at 20 min compared with 3 min in tests with chickens ($t = 9.91$, df = 15, $P < 0.0001$), CO_2 ($t = 7.71$, df = 15, $P < 0.0001$), and the arm ($t = 2.30$, df = 15, $P = 0.01$) but not feathers ($t = 1.60$, df = 15, $P = 0.06$) (Fig. 2). Increases in collections ranged from 4.6% (feathers) to 29.4% (chicken). The increased duration of assays also resulted in significant increases at 20 min compared with 3 min in numbers of *Cx. quinquefasciatus* collected in response to chickens ($t = 4.36$, df = 15, $P < 0.001$), CO_2 ($t = 12.4$, df = 15, $P < 0.0001$), and

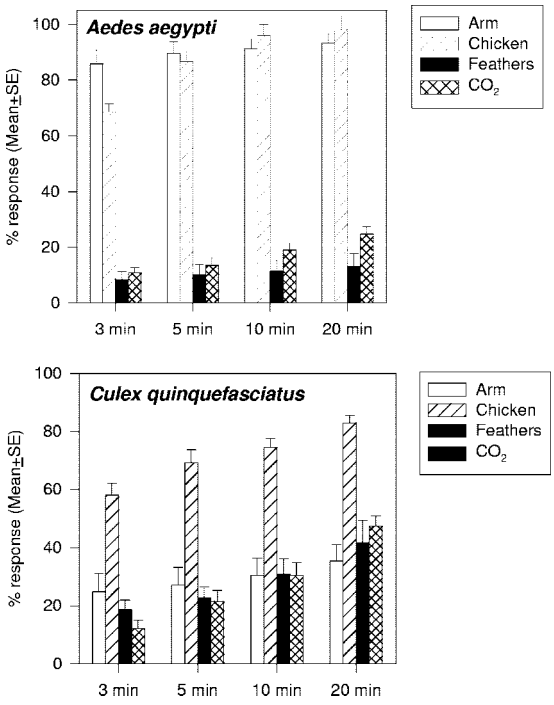

Fig. 2. Effect of assay duration on responses of host-seeking *Ae. aegypti* and *Cx. quinquefasciatus* to various treatments in a dual-port olfactometer. Bars represent percentage of total mosquitoes tested that were trapped in the treatment port.

feathers ($t = 3.76$, df = 15, $P < 0.001$). There was no difference in collections in response to arms ($t = 1.27$, df = 15, $P = 0.1$). Increases in collections ranged from 10.7% (arm) to 35.2% (CO_2). Because of the increased collections, longer assays were used for evaluation of standard compounds and extracts.

Volatile compounds identified from feathers and the most common diols from uropygial gland extracts did not elicit attraction of *Cx. quinquefasciatus* (Table 1) with no differences between response to treatment or control ports. In addition, a mixture of these compounds in the proportions present in Williams et al. (2003) also did not attract mosquitoes (Table 2). Significant attraction of females in the olfactometer only occurred in the presence of rubbed cotton balls and hexane extracts (Table 2). Nonpolar ether extracts of feathers were not attractive. Attraction to extracts were moderate (16.2–30.7%) and significantly lower than to a chicken (82.9 ± 2.54) ($P > 0.05$). Feathers did not differ in level of attraction compared with rubbed cotton balls ($t = 0.81$, df = 22, $P < 0.21$) and the hexane extracts of feathers ($t = 0.97$, df = 22, $P < 0.17$).

Discussion

The *Culex* species tested in the olfactometer exhibited a strong preference for attraction to a chicken over the human arm. The most extreme preference for

Table 1. Attraction of *Cx. quinquefasciatus* females to volatile compounds previously identified from feathers and uropygiol standards in 20-min dual-port olfactometer assays

Compound	% mosquitoes attracted (±SE)	
	Treatment	Control
Hexanal	0.3 (0.2)a	2.0 (0.6)a
α-Pinene	4.8 (1.1)a	2.4 (0.6)a
Benzaldehyde	2.3 (1.2)a	0.7 (0.5)a
β-Myrcene	0.9 (0.6)a	0.0 (0.0)a
Nonanal	0.7 (0.3)a	1.7 (0.3)a
Mixture[a]	1.5 (0.2)a	3.1 (0.5)a
meso-2,3-Butanediol	0.7 (0.3)a	0.4 (0.3)a
2,3-Butanediol	6.5 (1.9)a	2.1 (0.7)a
2,3-Docosanediol	0.5 (0.2)a	0.2 (0.1)a

Means in each row followed by the same letter are not significantly different (paired *t*-test, $P < 0.05$) ($n = 12$).

[a] Mixture of 21% hexanal, 12% α-pinene, 12% benzaldehyde, 23% β-myrcene, and 32% nonanal based on Williams et al. (2003).

birds in the olfactometer was from *Cx. tarsalis*, which showed no attraction to the human arm. McIver (1968) also reported that *Cx. tarsalis* not to be attracted to a human arm compared with a chicken and concluded that this species was mostly attracted to CO_2 and possibly water vapor. The feeding pattern of *C. tarsalis* in the field was considered by Templis and Washino (1967) to be greatly influenced by host availability and season, and with a choice this species would be attracted to the host producing the most CO_2. This observation is in agreement with field choice trial results from Walters et al. (1979), indicating that more *Cx. tarsalis* were more attracted to a human host that to pigeon hosts. These reports of the importance of CO_2 in host attraction of *Cx. tarsalis* were supported in our study by the high levels of response by this species to CO_2 in the absence of other host stimuli. The low response of *Cx. tarsalis* to the human hand could possibly be because of the low levels of CO_2 present (Frame et al. 1972) or the presence of L-lactic acid that is present in high quantities in human skin but low quantities (>150-fold lower) on chicken skin (Dekker et al. 2002). This compound mediates the selection by *Anopheles gambiae* Giles of humans as favorable hosts and when presented in conjunction with cow odor repelled zoophilic tsetse (Vale 1979).

Another species with little response to the arm but a strong response to the chicken is *Cx. nigripalpus.* This species feeds opportunistically on mammals and avians, but it does not consider humans a particularly attractive host (Provost 1969). Another opportunistic

Table 2. Responses of *Cx. quinquefaciatus* females to feather extracts in-20 min dual-port olfactometer assays

	% mosquitoes attracted (±SE)	
	Treatment	Control
Rubbed cotton balls	32.7 (8.0)a	1.9 (0.1)b
Ether extract of feathers	1.3 (0.9)a	0.0 (0.0)a
Hexane extract of feathers	30.7 (5.6)a	0.0 (0.0)b

Means in each row followed by the same letter are not significantly different (paired *t*-test, $P < 0.05$) ($n = 12$).

species, *Cx. quinquefasciatus*, is thought to feed readily on birds as well as mammals, although it is often considered to have preference for avians as based on bloodmeal identifications (Hayes et al. 1973, Reisen et al. 1990). *Ae. aegypti* is generally considered to feed opportunistically (Clements 1999) with equal attraction of females to human arms and chicks in a laboratory test (McIver 1968).

Carbon dioxide is important in activation of mosquitoes and in the presence of host-related odors, a strong attraction response may be observed with considerable differences in responses between species (Gillies 1980, Clements 1999). Carbon dioxide was considered essential for attraction indicated by flight behavior to lactic acid with little response to lactic acid alone (Smith et al. 1970, Price et al. 1979). Various reports indicate that CO_2 acts in a synergistic fashion in conjunction with other host odors such as 1-octen-3-ol (Takken and Kline 1989; Kline et al. 1990, 1991) or an additive manner such as with lactic acid (Eiras and Jepson 1991). However, in other studies, CO_2 in combination with host odors was not more attractive than to CO_2 alone (Mboera et al. 1998). The relatively low levels of attraction to CO_2 alone in the olfactometer were similar to those previously published for *Ae. aegypti* (Eiras and Jepson 1991, Bernier et al. 2001) and *Cx. quinquefasciatus* (Omer 1979). Mboera et al. (1998) reported that *Cx. quinquefasciatus* was poorly attracted to CO_2 at levels present in human breath. Similar to previous studies with humans and guinea pigs (Bar-zeev 1977, Bos and Laarman 1975), our study found that host odor (feathers in our study) in combination with CO_2 elicited the strongest behavioral responses, followed by CO_2 and host odor alone. The addition of CO_2 to the feather volatiles enhanced attractiveness but not to the level of attraction of a chicken for all mosquito species. The greater response to the chicken may be because of additional attractants from chicken breath, emanations from the skin, loss of important volatile compounds during the feather extraction process, or lack of appropriate concentrations or mixtures of compounds.

The uropygial or preen gland is a sebaceous gland of birds and plays a role in waterproofing feathers by the act of preening or distributing waxes over the feathers (Moyer et al. 2003). This gland, however, may not be the only source of waxes on the feathers, and other compounds associated with keratinization (Bollinger and Varga 1961) and possibly epidermal glands in feather follicles (Sandilands et al. 2004) also may contribute to the materials present on feathers. Patterns of lipid constituents in uropygial glands of birds are characteristic for a species with considerable and variation between taxa (Jacob and Ziswiler 1982). Uropygial gland secretions are considered the main sources of avian integumental lipids, and in most circumstances, they consist of monoester waxes containing fatty acid and monohydric alcoholic moieties. In some avian groups such as galliform birds, diols substitute for the monohydric alcohols with the characteristic presence of alkane-2,3-diols (Jacob 1992). In chickens, uropygial gland secretions consist of as much as 97% of diester waxes of 2,3-alkanediols from C19–C23. Some of the remaining compounds present include triacylglycerols, cholesterol, and phospholipids (Haahti and Fales 1967, Hansen et al. 1969). Much of the current literature on feather waxes focuses on compounds useful in avian taxonomic and phylogenetic studies (Jacob and Ziswiler 1982, Jacob 1992, Sweeney et al. 2004) and does not necessarily include compounds that may be volatile and used for mosquito location of avian hosts.

Feathers are important in eliciting attraction of *Culex* mosquitoes. The role of birds in attraction of biting flies was elucidated by Fallis and Smith (1964) who reported that ether extracts of loons, but not other waterfowl species, attracted simuliids. Zeman (1988) reported that benzene (nonpolar), but not ether (moderately polar) extracts of feathers, elicited significant feeding responses of poultry red mites on extract-treated membrane feeders. Additionally, responses of feather extracts were almost double those of hen uropygial gland secretion. Our results with hexane extracts containing attractants are similar to those of Zeman (1988). None of the chemical standards identified by Williams et al. (2003) or Haahti and Fales (1967) evaluated in this study elicited attraction in the olfactometer, but further studies are needed to identify the compounds or mixtures of compounds responsible for mosquito attraction to feathers. Solvent extracts of feathers were effective in eliciting attraction of mosquitoes and likely contain several volatile compounds that play a role in mosquito attraction. These extracts will be the basis for our future studies on identification of the volatile compounds from feathers that elicit attraction of *Culex* mosquitoes.

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